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PATENT APPLICATION

**METHODS FOR ALTERING ORGAN MASS, CONTROLLING
FERTILITY AND ENHANCING ASEXUAL REPRODUCTION IN
PLANTS**

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5 **METHODS FOR ALTERING ORGAN MASS, CONTROLLING
FERTILITY AND ENHANCING ASEXUAL REPRODUCTION IN
PLANTS**

CROSS REFERENCE TO RELATED APPLICATIONS

This application is related to USSN 09/227,421, filed January 8, 1999,
10 which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention is directed to plant genetic engineering. In
particular, it relates to methods of altering organ mass, controlling fertility and enhancing
15 asexual reproduction in plants through the modulation of plant growth and cell
proliferation.

BACKGROUND OF THE INVENTION

Control of organ mass/size and fertility in plants is a significant goal in
20 commercial agriculture. Plant shoot vegetative organs and/or structures (*e.g.* leaves,
stems and tubers), roots, flowers and floral organs (*e.g.* bracts, sepals, petals, stamens,
carpels, anthers), ovules (including egg and central cells), seed (including zygote,
embryo, endosperm, and seed coat), fruit (the mature ovary) and seedlings are the
harvested product of numerous agronomically-important crop plants. Therefore the
25 ability to manipulate the size/mass of these organs/structures through genetic control
would be an important agricultural tool. Similarly, induction of sterility in plants is useful
in limiting plant pollination and reproduction until it is economically desirable. For
example, male sterile plants are often desirable in crops where hybrid vigor increases
yield.

The intrinsic plant organ size is determined genetically, although it can be altered greatly by environment signals (e.g. growth conditions). In general, larger organs consist of larger numbers of cells. Since neither cell migration nor cell death plays a major role during plant development, the number of cells in plant organs depends on cell proliferation. Precise regulation of cell proliferation is also necessary for proper development of reproductive organs that make plants fertile. While some basic research has identified genes involved in plant organ development and fertility, little is known about genetic control of cell proliferation or its link to organogenesis including organ size/mass control and fertility in plants. Therefore an important goal is to understand the connection between genes that control organogenesis and genes that control cell proliferation. A great deal of basic research has shown that the components (e.g., cyclin dependent kinases, cyclins and their inhibitors) and mechanisms (e.g., regulation by phosphorylations, ubiquitin-mediated proteolysis) that control the cell cycle in yeast and animals are conserved in higher plants (Burssens, *et al. Plant Physiol Biochem.* 36:9-19 (1998)).

In *Arabidopsis*, the developing flower includes the ovule. Wild-type ovule development in *Arabidopsis* has been extensively analyzed (Robinson-Beers *et al.*, *Plant Cell* 4:1237-1249 (1992); Modrusan, *et al. Plant Cell.* 6:333-349 (1994) and Schneitz *et al.*, *Plant J.* 7:731-749 (1995)). A variety of mutations that affect ovule development have been identified (Klucher *et al.*, *Plant Cell* 8:137-153 (1996); Elliott *et al.*, *Plant Cell.* 8:155-168 (1996); Baker, *et al.*, *Genetics.* 145:1109-1124 (1997); Robinson-Beers, *et al.*, *Plant Cell.* 4:1237-1249 (1992); Modrusan *et al. Plant Cell.* 6:333-349 (1994); Ray, A., *et al. Proc Natl Acad Sci USA.* 91:5761-5765 (1994); Lang, *et al.*, *Genetics* 137:1101-1110 (1994); Leon-Kloosterziel *Plant Cell.* 6:385-392 (1994); Gaiser *et al.*, *Plant Cell* 7:333-345 (1995)), and some of them have been found that specifically affect patterns of cell division (Schneitz, *et al. Development.* 124:1367-1376 (1997)). Of those, several genes have been cloned; *AINTEGUMENTA (ANT)* (Klucher *et al. Plant Cell.* 8:137-153 (1996); Elliott *et al.*, *Plant Cell.* 8:155-168 (1996)), *AGAMOUS*, (Yanofsky *et al.*, *Nature.* 346:35-39 (1990); Bowman *et al.*, *Plant Cell.* 3:749-758 (1991)), *SUPERMAN* (Sakai *et al.*, *Nature.* 378:199-203 (1995)). Because these genes are expressed and function not only in developing ovules but also in various developing

organs, analysis of these mutations and genes has provided general information about the control of cell proliferation during plant development.

Another trait important to the manipulation of crop species is the ability to reproduce or propagate plants through asexual means, particularly vegetative propagation of sterile or hybrid plants, and regeneration of plants from transformed cells. Asexual reproduction includes regeneration of plants from cells or tissue, propagation of plants through cutting by inducing adventitious shoots and roots, and apomixis by forming somatic embryos. Asexual reproduction has the advantage that genetic clones of plants with desirable traits can be readily produced. Not all plants, however, can produce adventitious shoots or roots, or regenerate whole plants from cells or tissue.

In spite of the recent progress in defining the genetic control of plant cell proliferation, little progress has been reported in the identification and analysis of genes effecting agronomically important traits such as organ mass/size, fertility, asexual reproduction, and the like through regulating cell proliferation. Characterization of such genes would allow for the genetic engineering of plants with a variety of desirable traits. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

The present invention provides methods for modulating cell proliferation and thus cell number in plants by modulating *ANT* activity in plants. Typically, the methods comprise modulating the expression of *ANT* in plants and selecting for plants with altered size/mass, fertility, or both. In some preferred embodiments, the *ANT* activity is increased and plants with increased cell proliferation and thus increased cell number are selected. One method for modulating *ANT* expression is by introducing into a plant an expression cassette containing a heterologous *ANT* nucleic acid operably linked to a promoter. Examples of possible *ANT* nucleic acids that can be used include nucleic acids at least 50% identical to SEQ ID NO:1 and SEQ ID NO:4. Other examples include nucleic acids that encode the polypeptides at least 60% identical to either SEQ ID NO:2 or SEQ ID NO:5.

The present invention also provides methods for modulating cell proliferation and thus the production of adventitious organs in plants. Typically, the methods comprise increasing the activity or expression of *ANT* in plants and selecting for

plants with adventitious shoots, organs or structures such as embryos. One method for modulating *ANT* expression is by introducing into a plant an expression cassette containing a heterologous *ANT* nucleic acid operably linked to a promoter. Examples of possible *ANT* nucleic acids that can be used include nucleic acids at least 50% identical to
5 SEQ ID NO:1 and SEQ ID NO:4. Other examples include nucleic acids that encode the polypeptides at least 60% identical to either SEQ ID NO:2 or SEQ ID NO:5.

The present invention also provides methods of reproducing a plant through asexual means. Typically, the methods comprise increasing the activity or expression of *ANT* in plants and selecting a plant reproduced from the plant cell or tissue.

10 One method for modulating *ANT* expression is by introducing into a plant an expression cassette containing a heterologous *ANT* nucleic acid operably linked to a promoter. Examples of possible *ANT* nucleic acids that can be used include nucleic acids at least 50% identical to SEQ ID NO:1 and SEQ ID NO:4. Other examples include nucleic acids that encode the polypeptides at least 60% identical to either SEQ ID NO:2 or SEQ ID
15 NO:5. In various embodiments, the plant arises from an adventitious shoot, a somatic embryo, or a cutting.

In another embodiment of the invention, a heterologous gene is expressed in meristematic tissue of a plant by introducing into a plant an expression cassette containing an *ANT* promoter operably linked to a heterologous polynucleotide. In a
20 preferred embodiment of this invention, the *ANT* promoter is shown in SEQ ID NO:3.

The invention also provides isolated nucleic acid molecules comprising an *ANT* nucleic acid that specifically hybridizes to SEQ ID NO: 4, which is isolated from *Brassica napus*.

A variety of plant promoters can be used in the methods of the invention.

25 The promoter can be constitutive, inducible or specific for an organ, tissue, or cell. In some embodiments a promoter from an *ANT* gene, e.g. SEQ ID NO: 3, is used. Expression of the *ANT* nucleic acids of the invention can be directed to any desired organ, tissue, or cell in the plant. In some preferred embodiments of the invention, the promoter directs expression of the *ANT* nucleic acid in shoot vegetative organs/structures, such as
30 leaf, stem and tuber. In other preferred embodiments, the promoter directs expression of the *ANT* nucleic acid in roots. In other preferred embodiments, the promoter directs expression of the *ANT* nucleic acid in flowers or floral organs/structures, such as bracts,

sepals, petals, stamens, carpels, anthers and ovules. In different embodiments, the promoter directs expression of the *ANT* nucleic acid in seeds (e.g. embryo, endosperm, and seed coat) or fruit.

5 Definitions

The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role.

10 The term "promoter" refers to regions or sequence located upstream and/or downstream from the start of transcription and which are involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells.

The term "plant" includes whole plants, shoot vegetative organs and/or
15 structures (e.g. leaves, stems and tubers), roots, flowers and floral organs (e.g. bracts, sepals, petals, stamens, carpels, anthers), ovules (including egg and central cells), seed (including zygote, embryo, endosperm, and seed coat), fruit (e.g., the mature ovary), seedlings, plant tissue (e.g. vascular tissue, ground tissue, and the like), cells (e.g. guard cells, egg cells, trichomes and the like), and progeny of same. The class of plants that can
20 be used in the method of the invention is generally as broad as the class of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and multicellular algae. It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous.

25 "Increased or enhanced *ANT* activity or expression of the *ANT* gene" refers to an augmented change in *ANT* activity. Examples of such increased activity or expression include the following. *ANT* activity or expression of the *ANT* gene is increased above the level of that in wild-type, non-transgenic control plants (i.e. the quantity of *ANT* activity or expression of the *ANT* gene is increased). *ANT* activity or
30 expression of the *ANT* gene is in an organ, tissue or cell where it is not normally detected in wild-type, non-transgenic control plants (i.e. spatial distribution of *ANT* activity or expression of the *ANT* gene is increased). *ANT* activity or expression is increased when

ANT activity or expression of the *ANT* gene is present in an organ, tissue or cell for a longer period than in a wild-type, non-transgenic controls (i.e. duration of ANT activity or expression of the *ANT* gene is increased).

As used herein, the term “asexual reproduction” refers to the formation of shoots, roots or a whole plant from a plant cell without fertilization. If the formation of the whole plant proceeds through a somatic embryo, the asexual reproduction can be referred to as apomixis.

The term “adventitious organ” and “adventitious shoot” refer to an organ (e.g. stem, leaf, or root) and a shoot arising in a place other than its usual site, respectively. For example, a root developing on a stem, or a shoot bud arising on a stem in a place other than the axil of a leaf. Adventitious organs or shoots may also arise in callus tissue *in vitro*. Such adventitious organs or shoots can then be used to regenerate a whole plant using methods well known to those of skill in the art.

A polynucleotide sequence is “heterologous to” an organism or a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is not naturally associated with the promoter (e.g. a genetically engineered coding sequence or an allele from a different ecotype or variety).

A polynucleotide “exogenous to” an individual plant is a polynucleotide which is introduced into the plant by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation, biolistic methods, electroporation, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T₁ (e.g. in *Arabidopsis* by vacuum infiltration) or R₀ (for plants regenerated from transformed cells *in vitro*) generation transgenic plant. Transgenic plants that arise from sexual cross or by selfing are descendants of such a plant.

An “*ANT* nucleic acid” or “*ANT* polynucleotide sequence” of the invention is a subsequence or full length polynucleotide sequence (SEQ ID NO:1) which, encodes a polypeptide (SEQ ID NO:2) and its complement, as described, for instance, by Klucher *et al.*, *Plant Cell* 8:137-153 (1996) and Elliott *et al.*, *Plant Cell*. 8:155-168 (1996) (*see, also*,

GenBank Accession Nos. U40256 and U41339). SEQ ID NO:4, which encodes SEQ ID NO:5, represents another "ANT nucleic acid" from *Brassica*. ANT gene products of the invention are characterized by the presence of an AP2 domain, first identified in AP2, this motif is characterized by a region of approximately 60-70 amino acid residues with a highly conserved core region with the capacity to form an amphipathic α -helix and/or to bind DNA (Jofuku *et al.*, *Plant Cell* 6:1211-1225 (1994); Ohme-Takagi and Shinshi, *Plant Cell* 7: 173-182 (1995). The full length ANT protein contains two AP2 domains (amino acids 281 to 357 and from 383 to 451 of SEQ ID NO:2) and a linker region (amino acids 358 to 382), and the homology to other AP2 domain proteins is restricted to this region. An ANT polynucleotide of the invention typically comprises a coding sequence at least about 30-40 nucleotides to about 2500 nucleotides in length, usually less than about 3000 nucleotides in length. Usually the ANT nucleic acids of the invention are from about 100 to about 5000 nucleotides, often from about 500 to about 3000 nucleotides in length.

In the case of both expression of transgenes and inhibition of endogenous genes (e.g., by antisense, or co-suppression) one of skill will recognize that the inserted polynucleotide sequence need not be identical, but may be only "substantially identical" to a sequence of the gene from which it was derived. As explained below, these substantially identical variants are specifically covered by the term ANT nucleic acid.

In the case where the inserted polynucleotide sequence is transcribed and translated to produce a functional polypeptide, one of skill will recognize that because of codon degeneracy a number of polynucleotide sequences will encode the same polypeptide. These variants are specifically covered by the terms "ANT nucleic acid", "ANT polynucleotide" and their equivalents. In addition, the terms specifically include those full length sequences substantially identical (determined as described below) with an ANT polynucleotide sequence and that encode proteins that retain the function of the ANT polypeptide (e.g., resulting from conservative substitutions of amino acids in the ANT polypeptide).

The term "altered fertility" includes any transient or permanent alteration of fecundity including inducing sterility as well as altered initiation of floral

development (e.g., flowering time). Sterility can be caused, *inter alia*, by disruption of pollen development, dehiscence (i.e. male sterility), by disruption of ovule development (i.e. female sterility), or by disruption of pollination /fertilization processes caused by abnormal development of male/female organs (e. g. stigmatic papillae, transmitting tissue of septum). Flowering time is the developmental time or stage when a plant initiates and produces floral tissue.

Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated according to, e.g., the algorithm of Meyers & Miller, *Computer Applic. Biol. Sci.* 4:11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to a sequence or subsequence that has at least 25% sequence identity with a reference sequence. Alternatively, percent identity can be any integer

from 25% to 100%. More preferred embodiments include at least: 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%. compared to a reference sequence using the programs described herein; preferably BLAST using standard parameters, as described below. This definition also refers to the complement of a test sequence, when the test sequence has substantial identity to a reference sequence.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by

Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al, supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA*

89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g., Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.01, more preferably less than about 10^{-5} , and most preferably less than about 10^{-20} .

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, in a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an

amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

- 5 1) Alanine (A), Serine (S), Threonine (T);
 - 2) Aspartic acid (D), Glutamic acid (E);
 - 3) Asparagine (N), Glutamine (Q);
 - 4) Arginine (R), Lysine (K);
 - 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
 - 10 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).
- (see, e.g., Creighton, *Proteins* (1984)).

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide
15 encoded by the second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below.

20 The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions
25 under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--*
30 *Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, highly stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific

sequence at a defined ionic strength pH. Low stringency conditions are generally selected to be about 15-30 °C below the T_m . The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 time background hybridization.

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions.

In the present invention, genomic DNA or cDNA comprising *ANT* nucleic acids of the invention can be identified in standard Southern blots under stringent conditions using the nucleic acid sequences disclosed here. For the purposes of this disclosure, suitable stringent conditions for such hybridizations are those which include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and at least one wash in 0.2X SSC at a temperature of at least about 50°C, usually about 55°C to about 60°C, for 20 minutes, or equivalent conditions. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

A further indication that two polynucleotides are substantially identical is if the reference sequence, amplified by a pair of oligonucleotide primers, can then be used as a probe under stringent hybridization conditions to isolate the test sequence from a cDNA or genomic library, or to identify the test sequence in, e.g., an RNA gel or DNA gel blot hybridization analysis.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention relates to control of cell proliferation and thus cell number in plants by modulating *ANT* activity in plants. For example, the invention provides molecular strategies for manipulating plant biomass through controlling the number of cells and size/mass of plant shoot vegetative organs and/or structures (e.g. leaves, stems and tubers), roots, flowers and floral organs (e.g. bracts, sepals, petals, stamens, carpels, anthers), ovules (including egg and central cells), seed (including zygote, embryo, endosperm, and seed coat), fruit (the mature ovary) and seedlings using *ANT* gene constructs. Thus, by regulating *ANT* expression transgenic plants with increased or decreased biomass can be produced. In addition, modulating expression of the gene in male or female reproductive organs can lead to sterility through alteration in the pattern of cell proliferation. Thus, male or female sterile transgenic plants can be produced by enhancing or inhibiting *ANT* gene expression in the appropriate tissues. In yet other embodiments, formation of adventitious organs, shoots, or structures such as somatic embryos can be controlled using this method of the invention. Thus, the efficiency of asexual reproduction of plants, in particular reproduction of sterile or hybrid plants with desired traits and regeneration of transgenic plants from transformed tissue, can be improved.

Because the *ANT* gene product most likely functions as a transcription factor (Vergani et al., *FEBS Letters*. 400:243-246 (1997)), one of skill will recognize that desired phenotypes associated with altered *ANT* activity can be obtained by modulating the expression or activity of *ANT*-regulated genes. Any of the methods described for increasing or decreasing *ANT* expression or activity can be used for this purpose.

Increasing ANT activity or *ANT* gene expression

Any of a number of means well known in the art can be used to increase ANT activity in plants. Enhanced expression is useful, for example, to induce or enhance asexual reproduction, or increase organ size/mass in desired plant organs. Any organ can be targeted, such as Plant shoot vegetative organs and/or structures (e.g. leaves, stems and tubers), roots, flowers and floral organs (e.g. bracts, sepals, petals, stamens, carpels, anthers), ovules (including egg and central cells), seed (including zygote, embryo, endosperm, and seed coat), fruit and seedlings. The beneficial effects of altering ANT activity need not be the direct result of increased cell proliferation. For instance, increased leaf size/mass will lead to an increase in photosynthesis, which will in turn lead to increased yield. Similarly increased mass/ size of roots will lead to increased nutrient uptake and increased yield. Increased stem or pedicel thickness can be used to decrease losses due to breakage, e.g. in cereal crops and fruits.

Increased ANT activity or *ANT* expression can also be used to produce male or female sterile plants. Male or female sterility is important for agriculture and horticulture, particularly in the production of hybrid varieties that have commercially advantageous superior traits. Male or female sterility allows breeders to make hybrid varieties easily by preventing self-pollen contamination in parental plants. Thus, for instance targeting *ANT* expression in developing anthers will cause male sterility, but not disrupt female organs thus rendering plants female fertile. Prevention of dehiscence is also desirable for commercial cut flowers. For instance, pollination leads to floral senescence, also pollen grains can be allergenic and in some plants (e.g., lilies) can cause stains.

Expression of the *ANT* gene in transgenic plants can also cause female sterility. Plants constitutively expressing the *ANT* gene in ovules produce large mature ovules that are sterile. Therefore, introducing female sterility via controlling *ANT* function can delay senescence of plants and improve vegetative yield and quality of crop and horticulturally important plants. Alternatively, female sterility can result from decreased *ANT* expression using methods described below for methods of inhibiting ANT activity or gene expression.

Increasing *ANT* gene expression

Isolated sequences prepared as described herein can be used to introduce expression of a particular *ANT* nucleic acid to increase endogenous gene expression using methods well known to those of skill in the art. Preparation of suitable constructs and means for introducing them into plants are described below.

One of skill will recognize that the polypeptides encoded by the genes of the invention, like other proteins, have different domains that perform different functions. Thus, the gene sequences need not be full length, so long as the desired functional domain of the protein is expressed. The distinguishing features of *ANT* polypeptides, including the AP2 domain, nuclear localization signal, and transcription activation domains, are discussed in Elliot *et al.* or Klucher *et al.* above.

Modified protein chains can also be readily designed utilizing various recombinant DNA techniques well known to those skilled in the art and described in detail, below. For example, the chains can vary from the naturally occurring sequence at the primary structure level by amino acid substitutions, additions, deletions, and the like. These modifications can be used in a number of combinations to produce the final modified protein chain.

Modification of endogenous *ANT* genes

Methods for introducing genetic mutations into plant genes and selecting plants with desired traits are well known. For instance, seeds or other plant material can be treated with a mutagenic chemical substance, according to standard techniques. Such chemical substances include, but are not limited to, the following: diethyl sulfate, ethylene imine, ethyl methanesulfonate and N-nitroso-N-ethylurea. Alternatively, ionizing radiation from sources such as, X-rays or gamma rays can be used.

Alternatively, homologous recombination can be used to induce targeted gene modifications by specifically targeting the *ANT* gene *in vivo* (see, generally, Grewal and Klar, *Genetics* 146: 1221-1238 (1997) and Xu *et al.*, *Genes Dev.* 10: 2411-2422 (1996)). Homologous recombination has been demonstrated in plants (Puchta *et al.*, *Experientia* 50: 277-284 (1994), Swoboda *et al.*, *EMBO J.* 13: 484-489 (1994); Offringa *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 7346-7350 (1993); and Kempin *et al.* *Nature* 389:802-803 (1997)).

In applying homologous recombination technology to the genes of the invention, mutations in selected portions of an *ANT* gene sequences (including 5' upstream, 3' downstream, and intragenic regions) such as those disclosed here are made *in vitro* and then introduced into the desired plant using standard techniques. Since the efficiency of homologous recombination is known to be dependent on the vectors used, use of dicistronic gene targeting vectors as described by Mountford *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 4303-4307 (1994); and Vaulont *et al.*, *Transgenic Res.* 4: 247-255 (1995) are conveniently used to increase the efficiency of selecting for altered *ANT* gene expression in transgenic plants. The mutated gene will interact with the target wild-type gene in such a way that homologous recombination and targeted replacement of the wild-type gene will occur in transgenic plant cells, resulting in suppression of ANT activity.

Alternatively, oligonucleotides composed of a contiguous stretch of RNA and DNA residues in a duplex conformation with double hairpin caps on the ends can be used. The RNA/DNA sequence is designed to align with the sequence of the target *ANT* gene and to contain the desired nucleotide change. Introduction of the chimeric oligonucleotide on an extrachromosomal T-DNA plasmid results in efficient and specific *ANT* gene conversion directed by chimeric molecules in a small number of transformed plant cells. This method is described in Cole-Strauss *et al.* *Science* 273:1386-1389 (1996) and Yoon *et al.* *Proc. Natl. Acad. Sci. USA* 93: 2071-2076 (1996).

Other means for increasing ANT activity

One method to increase *ANT* expression is to use "activation mutagenesis" (*see, e.g. Hiyashi et al. Science* 258:1350-1353 (1992)). In this method an endogenous *ANT* gene can be modified to be expressed constitutively, ectopically, or excessively by insertion of T-DNA sequences that contain strong/constitutive promoters upstream of the endogenous *ANT* gene. As explained below, preparation of transgenic plants overexpressing *ANT* can also be used to increase *ANT* expression. Activation mutagenesis of the endogenous *ANT* gene will give the same effect as overexpression of the transgenic *ANT* nucleic acid in transgenic plants. Alternatively, an endogenous gene encoding an enhancer of ANT activity or expression of the endogenous *ANT* gene can be modified to be expressed by insertion of T-DNA sequences in a similar manner and ANT activity can be increased.

Another strategy to increase *ANT* expression can be the use of dominant hyperactive mutants of *ANT* by expressing modified *ANT* transgenes. For example expression of modified *ANT* with a defective domain that is important for interaction with a negative regulator of *ANT* activity can be used to generate dominant hyperactive *ANT* proteins. Alternatively, expression of truncated *ANT* proteins which have only a domain that interacts with a negative regulator can titrate the negative regulator and thereby increase endogenous *ANT* activity. Use of dominant mutants to hyperactivate target genes is described in Mizukami *et al. Plant Cell* 8:831-845 (1996).

10 Inhibition of *ANT* activity or gene expression

As explained above, *ANT* activity is important in controlling a number of plant processes through the regulation of cell proliferation. Inhibition of *ANT* gene expression activity can be used, for instance, to decrease plant organ size/mass or to induce female sterility in plants. In particular, targeted expression of *ANT* nucleic acids that inhibit endogenous gene expression (*e.g.*, antisense or co-suppression) can be used to inhibit ovule development at early stages and thus induce female sterility. The life span of the transgenic plants can therefore be extended because fertilization (seed formation) can activate and accelerate senescence processes of plants or organs.

Inhibition of *ANT* gene function can also be used to truncate vegetative growth, resulting in early flowering. Methods that control flowering time are extremely valuable in agriculture to optimize harvesting time as desired. Therefore, by regulating the function of the *ANT* genes in plants, it is possible to control time of flowering. For instance, acceleration of fertile plant growth can be obtained by expressing *ANT* antisense RNA during vegetative development to achieve early flowering. Expression of the *ANT* transgene can then be shut off during reproductive development to get fertile plants.

Inhibition of *ANT* gene expression

The nucleic acid sequences disclosed here can be used to design nucleic acids useful in a number of methods to inhibit *ANT* or related gene expression in plants. For instance, antisense technology can be conveniently used. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the antisense strand of RNA will be transcribed. The construct is then transformed into plants and the antisense strand of RNA is produced. In plant cells, it has

been suggested that antisense suppression can act at all levels of gene regulation including suppression of RNA translation (*see*, Bourque *Plant Sci. (Limerick)* 105: 125-149 (1995); Pantopoulos In Progress in Nucleic Acid Research and Molecular Biology, Vol. 48. Cohn, W. E. and K. Moldave (Ed.). Academic Press, Inc.: San Diego, California, USA; London, England, UK. p. 181-238; Heiser *et al. Plant Sci. (Shannon)* 127: 61-69 (1997)) and by preventing the accumulation of mRNA which encodes the protein of interest, (*see*, Baulcombe *Plant Mol. Bio.* 32:79-88 (1996); Prins and Goldbach *Arch. Virol.* 141: 2259-2276 (1996); Metzlaff *et al. Cell* 88: 845-854 (1997), Sheehy *et al., Proc. Nat. Acad. Sci. USA*, 85:8805-8809 (1988), and Hiatt *et al., U.S. Patent No.* 4,801,340).

The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous *ANT* gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other genes within a family of genes exhibiting identity or substantial identity to the target gene.

For antisense suppression, the introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides and about full length nucleotides should be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of about 500 to about 3500 nucleotides is especially preferred.

A number of gene regions can be targeted to suppress *ANT* gene expression. The targets can include, for instance, the coding regions, introns, sequences from exon/intron junctions, 5' or 3' untranslated regions, and the like.

Another well known method of suppression is sense co-suppression. Introduction of nucleic acid configured in the sense orientation has been recently shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes (*see*,

Assaad *et al. Plant Mol. Bio.* 22: 1067-1085 (1993); Flavell *Proc. Natl. Acad. Sci. USA* 91: 3490-3496 (1994); Stam *et al. Annals Bot.* 79: 3-12 (1997); Napoli *et al., The Plant Cell* 2:279-289 (1990); and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184).

The suppressive effect may occur where the introduced sequence
5 contains no coding sequence *per se*, but only intron or untranslated sequences
homologous to sequences present in the primary transcript of the endogenous sequence.
The introduced sequence generally will be substantially identical to the endogenous
sequence intended to be repressed. This minimal identity will typically be greater than
about 65%, but a higher identity might exert a more effective repression of expression of
10 the endogenous sequences. Substantially greater identity of more than about 80% is
preferred, though about 95% to absolute identity would be most preferred. As with
antisense regulation, the effect should apply to any other proteins within a similar family
of genes exhibiting identity or substantial identity.

For co-suppression, the introduced sequence, needing less than
15 absolute identity, also need not be full length, relative to either the primary transcription
product or fully processed mRNA. This may be preferred to avoid concurrent production
of some plants which are overexpressers. A higher identity in a shorter than full length
sequence compensates for a longer, less identical sequence. Furthermore, the introduced
sequence need not have the same intron or exon pattern, and identity of non-coding
20 segments will be equally effective. Normally, a sequence of the size ranges noted above
for antisense regulation is used. In addition, the same gene regions noted for antisense
regulation can be targeted using co-suppression technologies.

Oligonucleotide-based triple-helix formation can also be used to
disrupt *ANT* gene expression. Triplex DNA can inhibit DNA transcription and
25 replication, generate site-specific mutations, cleave DNA, and induce homologous
recombination (*see, e.g., Havre and Glazer J. Virology* 67:7324-7331 (1993); Scanlon *et al. FASEB J.* 9:1288-1296 (1995); Giovannangeli *et al. Biochemistry* 35:10539-10548
(1996); Chan and Glazer *J. Mol. Medicine (Berlin)* 75: 267-282 (1997)). Triple helix
DNAs can be used to target the same sequences identified for antisense regulation.

30 Catalytic RNA molecules or ribozymes can also be used to inhibit
expression of *ANT* genes. It is possible to design ribozymes that specifically pair with
virtually any target RNA and cleave the phosphodiester backbone at a specific location,

thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. Thus, ribozymes can be used to target the same sequences identified for antisense regulation.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs which are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Zhao and Pick, *Nature* 365:448-451 (1993); Eastham and Ahlering, *J. Urology* 156:1186-1188 (1996); Sokol and Murray, *Transgenic Res.* 5:363-371 (1996); Sun *et al.*, *Mol. Biotechnology* 7:241-251 (1997); and Haseloff *et al.*, *Nature*, 334:585-591 (1988).

Modification of endogenous ANT genes

Methods for introducing genetic mutations described above can also be used to select for plants with decreased ANT expression.

Other means for inhibiting ANT activity

ANT activity may be modulated by eliminating the proteins that are required for ANT cell-specific gene expression. Thus, expression of regulatory proteins and/or the sequences that control ANT gene expression can be modulated using the methods described here.

Another strategy is to inhibit the ability of an ANT protein to interact with itself or with other proteins. This can be achieved, for instance, using antibodies specific to ANT. In this method cell-specific expression of ANT-specific antibodies is used to inactivate functional domains through antibody:antigen recognition (*see*, Hupp *et al.* *Cell* 83:237-245 (1995)). Interference of activity of an ANT interacting protein(s) can be applied in a similar fashion. Alternatively, dominant negative mutants of ANT can be prepared by expressing a transgene that encodes a truncated ANT protein. Use of

dominant negative mutants to inactivate target genes in transgenic plants is described in Mizukami *et al. Plant Cell* 8:831-845 (1996).

Isolation of ANT nucleic acids

5 Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the
10 manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998).

15 The isolation of *ANT* nucleic acids may be accomplished by a number of techniques. For instance, oligonucleotide probes based on the sequences disclosed here can be used to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to
20 form concatemers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired organ, such as flowers, and a cDNA library which contains the *ANT* gene transcript is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissues in which *ANT* genes or homologs are expressed.

25 The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned *ANT* gene disclosed here. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. Alternatively, antibodies raised against an *ANT* polypeptide can be used to screen an mRNA expression library.

30 Alternatively, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of the *ANT* genes

directly from genomic DNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes. For a general overview of PCR see *PCR Protocols: A Guide to Methods and Applications*. (Innis, M, Gelfand, D., Sninsky, J. and White, T., eds.), *Academic Press*, San Diego (1990). Appropriate primers and probes for identifying *ANT* sequences from plant tissues are generated from comparisons of the sequences provided here (e.g. SEQ ID NO: 4) and those provided in Klucher *et al.* and Elliot *et al.*, *supra*.

Polynucleotides may also be synthesized by well-known techniques as described in the technical literature. See, e.g., Carruthers *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams *et al.*, *J. Am. Chem. Soc.* 105:661 (1983). Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence. Because at the very 5' and 3' ends the *Arabidopsis ANT* nucleotide sequence is very similar to the *Brassica ANT* nucleotide sequence but not to other *Arabidopsis* AP2-domain containing genes, the primers with nucleotide sequences shown in SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8 can be used to screen/isolate *ANT* orthologs in different species by RT-PCR.

Preparation of recombinant vectors

To use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, for example, Weising *et al.* *Ann. Rev. Genet.* 22:421-477 (1988). A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

For example, for overexpression, a plant promoter fragment may be employed which will direct expression of the gene in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation.

5 Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes known to those of skill. Such genes include for example, *ACT11* from *Arabidopsis* (Huang *et al. Plant Mol. Biol.* 33:125-139 (1996)), *Cat3* from *Arabidopsis* (GenBank No. U43147, Zhong *et al., Mol. Gen. Genet.* 251:196-203 (1996)), the gene encoding
10 stearyl-acyl carrier protein desaturase from *Brassica napus* (Genbank No. X74782, Solocombe *et al. Plant Physiol.* 104:1167-1176 (1994)), *GPc1* from maize (GenBank No. X15596, Martinez *et al. J. Mol. Biol.* 208:551-565 (1989)), and *Gpc2* from maize (GenBank No. U45855, Manjunath *et al., Plant Mol. Biol.* 33:97-112 (1997)).

15 Alternatively, the plant promoter may direct expression of the *ANT* nucleic acid in a specific tissue, organ or cell type (*i.e.* tissue-specific promoters) or may be otherwise under more precise environmental or developmental control (*i.e.* inducible promoters). Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, elevated temperature, the presence of
20 light, or sprayed with chemicals/hormones. One of skill will recognize that a tissue-specific promoter may drive expression of operably linked sequences in tissues other than the target tissue. Thus, as used herein a tissue-specific promoter is one that drives expression preferentially in the target tissue or cell type, but may also lead to some expression in other tissues as well.

25 A number of tissue-specific promoters can also be used in the invention. For instance, promoters that direct expression of nucleic acids in flowers, ovules, or anthers (particularly the tapetum) are useful in methods in which sterility is desired. An example of a promoter that directs expression in the ovule is the promoter from the *BEL1* gene described in Reiser *et al. Cell* 83:735-742 (1995) (GenBank No. U39944).
30 Examples of tapetal-specific promoters include TA29 from tobacco (Mariani *et al., Nature*, 347:737-41, (1990)), and A6 and A9 from *Brassica* (Paul *et al., Plant Mol. Biol.*, 19:611-22, (1992), Hird *et al. Plant Journal* 4:1023-1033 (1993)). Anther-specific

promoters could also be used such as ones isolated by Twell *et al.* (*Mol. Gen. Genet.*, 217:240-45, (1991)).

To introduce male sterility, the 2nd and 3rd floral organ (petal and stamens) -specific *AP3* promoter (Day, *et al.*, *Development* 121:2887, 1995), for example, can be used. The carpel specific *AGL1* (Flanagan and Ma, *Plant J.* 10:343, 1993) or *AGL5* (Savidge, *et al.*, *Plant Cell* 7:721, 1995) promoter can be applied for inducing female sterility only. Sterile plants, yet with increased perianth organs, can be obtained by constitutively expressing the *ANT* gene through *AG* promoter (Sieburth and Meyerowitz, *Plant Cell* 9:355, 1997) that is active only in reproductive organ primordia and developing male and female organs.

Using the *API* promoter (Gustafson-Brown, *et al.*, *Cell* 76:131, 1994) that is expressed in floral primordia at early stages of flower development and in developing perianth organs, fertile flowers with enlarged perianth organs can be produced. For the increase of aerial vegetative organ biomass, photosynthetic organ-specific promoters, such as the *RBCS* promoter (Khouidi, *et al.*, *Gene* 197:343, 1997), can be used. Root biomass can be increased by the constitutive *ANT* expression under the control of the root-specific *ANR1* promoter (Zhang & Forde, *Science*, 279:407, 1998). To increase seed size/mass (an agronomically import trait), seed-specific promoters, such as the *LEC* promoter (Lotan, *et al.*, *Cell* 93:1195 (1998)), the late-embryogenesis-abundant promoter (West *et al.* *Plant Cell* 6:173 (1994)), beta-conglycininin alpha-subunit promoter (West *et al.*), the lectin promoter (Goldberg *et al.* *Science* 266:605 (1994)), or the Kunitz trypsin inhibitor 3 promoter (Goldberg *et al.*) can be used. Any strong, constitutive promoters, such as the CaMV 35S promoter, can be used for the increase of total plant biomass.

If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences (*e.g.*, promoters or coding regions) from genes of the invention will typically comprise a marker gene that confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418,

bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

The present invention also provides promoter sequences from the *ANT* gene (SEQ ID NO:3), which can be used to direct expression of the *ANT* coding sequence or heterologous sequences in desired tissues. *ANT* is expressed in meristematic cells throughout the plant. *ANT* promoter sequences of the invention are therefore useful in targeting expression to meristematic cells in lateral roots, leaf primordia, developing leaves, floral primordia, floral organ primordia, developing floral organs, ovule primordia, developing ovules, developing embryos, and vascular systems. Genes whose expression can be targeted to these cells in immature organs include disease resistance genes, such as the *Arabidopsis NPR1* gene (Cao, *et al.*, *Cell* 88:57, 1997) and the nematode resistance locus *Gro1* and the *Phytophthora infestans* resistance locus *R7* of potato (Leister, *et al.*, *Nature Genetics* 14:421, 1996), for increasing resistance to pathogens and insects in young, sensitive organs.

Because the *ANT* promoter is expressed in developing embryos at late stages, some genes encoding regulators or key enzymes for biosynthesis of storage oils, proteins, or starches, such as *BiP* (Hatano, *et al.*, *Plant and Cell Physiology* 38:344, 1997), can be expressed by the control of the *ANT* promoter.

Production of transgenic plants

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al.* *Embo J.* 3:2717-2722 (1984). Electroporation techniques are described in Fromm *et al.* *Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein *et al.* *Nature* 327:70-73 (1987).

Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary vectors, are well described in the scientific literature. See, for example Horsch et al. *Science* 233:496-498 (1984), and Fraley et al. *Proc. Natl. Acad. Sci. USA* 80:4803 (1983) and *Gene Transfer to Plants*, Potrykus, ed. (Springer-Verlag, Berlin 1995).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired phenotype such as increased seed mass. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, MacMillan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. *Ann. Rev. of Plant Phys.* 38:467-486 (1987).

The nucleic acids of the invention can be used to confer desired traits on essentially any plant. Thus, the invention has use over a broad range of plants, including species from the genera *Anacardium*, *Arachis*, *Asparagus*, *Atropa*, *Avena*, *Brassica*, *Chlamydomonas*, *Chlorella*, *Citrus*, *Citrullus*, *Capsicum*, *Carthamus*, *Cocos*, *Coffea*, *Cucumis*, *Cucurbita*, *Cyrtomium*, *Daucus*, *Elaeis*, *Fragaria*, *Glycine*, *Gossypium*, *Helianthus*, *Heterocallis*, *Hordeum*, *Hyoscyamus*, *Lactuca*, *Laminaria*, *Linum*, *Lolium*, *Lupinus*, *Lycopersicon*, *Macrocystis*, *Malus*, *Manihot*, *Majorana*, *Medicago*, *Nereocystis*, *Nicotiana*, *Olea*, *Oryza*, *Osmunda*, *Panicum*, *Pennisetum*, *Persea*, *Phaseolus*, *Pistachia*, *Pisum*, *Pyrus*, *Polypodium*, *Prunus*, *Pteridium*, *Raphanus*, *Ricinus*, *Secale*, *Senecio*, *Sinapis*, *Solanum*, *Sorghum*, *Theobromus*, *Trigonella*, *Triticum*, *Vicia*, *Vitis*, *Vigna*, and *Zea*.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Using known procedures one of skill can screen for plants of the invention by detecting the increase or decrease of *ANT* mRNA or protein in transgenic plants. Means for detecting and quantitating mRNAs or proteins are well known in the art. The plants of the invention can also be identified by detecting the desired phenotype. For instance, increased biomass of organs or plants can be detected according to well-known techniques. Male or female sterility can be identified by testing for viable pollen and/or the ability to set seed.

The following Examples are offered by way of illustration, not limitation.

EXAMPLE 1

This example shows that increased *ANT* expression increases cell number and the size/mass of roots, leaves, floral organs, ovules and seeds in *Arabidopsis*.

An *ANT* cDNA with a *Bam*HI site right before the initiation codon of the *ANT* coding nucleotide sequence was created by PCR using synthetic oligonucleotide primers. This *ANT* nucleic acid (from C at 268 to T at 2148 (1881 nucleotides) from SEQ ID NO: 1) was ligated at the *Bgl*III site of the plasmid vector pMON530 (Rogers, *et al.*, *Meth. Enzymol.* 153:253, 1987) under the constitutive 35S promoter, and the recombinant plasmid DNA which has an insert of the *ANT* cDNA in the sense direction with respect to the *CaMV* 35S promoter (*35S::ANT*) were selected. *Agrobacterium* cells were transformed with the recombinant plasmid DNA, and used for *Agrobacterium*-mediated plant transformation by vacuum infiltration with *Arabidopsis* plants (Col-0 ecotype). T₁ seeds were collected from transformed plants about three weeks after vacuum infiltration, and planted on MS plates with kanamycin for screening T₁ transgenic seedlings.

T₁ seeds include oversized seeds, which were distinguished because they did not pass through a mesh of defined size. The majority of these seeds were

kanamycin resistant, carrying the 35S::ANT transgene. This phenotype was not observed in vector only controls.

Multiple T₁ seedlings were larger than vector only control transgenic seedlings. As they develop, T₁ plants produced a highly branched root system having a larger mass than vector only controls. In addition, the plants had enlarged leaves, floral organs, and ovules as compared to the vector only controls. For example, the average flower and leaf biomass of T₁ lines was approximately three times and 2.5 times that of the vector only control, respectively. DIC microscopy and scanning electron microscopy revealed that this enlarged organ phenotype of T₁ plants was due to the increased cell number in the organs. In addition, T₁ plants were sterile. Preliminary examination suggests that anthers fail to shed pollen (which are morphologically normal) and the ovules were unusually large with an increased number of nucellar cells that compress/displace the female gametophyte.

Because sterility made it difficult to generate and propagate homozygous transgenic lines, we used a chemical induction system as described by Aoyama, and Chua *Plant J.* 11:605-612 (1997) and McNellis *et al. Plant J.* 14:247-257 (1998) to regulate ectopic ANT transcription. This system utilizes a chimeric transcription factor gene (35S::GVG), consisting of the 35S promoter, the DNA-binding domain of the yeast transcription factor GAL4, a transactivating domain, and the receptor domain of the glucocorticoid receptor (GR). The ANT gene was inserted downstream from a promoter (UAS::ANT) containing the binding site for the GVG transcription factor. The 35S::GVG/UAS::ANT construct was introduced into wild-type *Arabidopsis* and fertile transgenic lines were obtained generally as described above.

Transgenic T₂ plants were germinated on MS agar plates and transferred to plates either with or without the chemical inducer, dexamethasone (DEX), a synthetic glucocorticoid hormone that binds and activates the GVG transcription factor. Multiple transgenic lines were obtained that displayed an enlarged leaf phenotype after treatment with DEX. The increase in organ size/mass is due to an increased number of cells. DEX had no effect on control transgenic plants with only the 35S::GVG/UAS vector. Taken together, these results suggest that ectopic ANT expression increases organ size/mass by increasing cell number.

Example 2

This example shows that essentially the same phenotypic changes observed in *Arabidopsis* were observed in tobacco.

For generating tobacco transgenic plants expressing *ANT* cDNA under the control of the constitutive 35S promoter, the above recombinant plasmid DNA was used for *Agrobacterium*-mediated tobacco callus transformation. Tobacco calli were induced from sterilized tobacco leaf (SR1 variety) placed on callus-inducing plates, then co-cultivated with *Agrobacterium* cells carrying the above recombinant DNA for three days. After washing bacterial cells out, leaf calli were placed on shoot-inducing agar plate containing kanamycin and carbenicillin to generate transformed shoots. These R₀ shoots were transferred on root inducing agar plates, then transplanted on soil after regeneration of roots. [deleted a sentence]

The R₀ plants in which the *ANT* gene was constitutively expressed under the control of the CaMV 35S promoter produced wider leaves (about 1.5 times that of vector only control transgenic plants), relatively larger flowers (about 1.7 time greater mass than vector only control transgenic plants), and sterility as observed with *Arabidopsis*. The sterility is largely caused by the failure of dehiscence of anthers as seen in the *Arabidopsis* transgenic anthers. Some R₀ plants produced functional pollen grains in their closed anthers, and produced seeds (R₁ seeds) upon self-pollination by hand using pollen grains dissected from the anther. These R₁ seeds had mass about 1.5 times that of seed from vector only control plants.

Example 3

This example describes plant organ size/mass reduction and altered flowering by co-suppressing endogenous gene activity by the *ANT* transgene in *Arabidopsis* and tobacco.

Arabidopsis T₁ lines described above included lines exhibiting reduced organ size/mass and organ cell numbers. These plants were completely or partially female sterile, as are loss-of-function *ant* mutants. In these lines, expression of *ANT* mRNA was highly reduced, suggesting that co-suppression of the endogenous *ANT* gene, as well as that of the *ANT* cDNA, took place in the lines. From partially sterile T₁ lines, transgenic T₂ plants were obtained that segregated for the same co-suppressed phenotype as in T₁ parental plants. Reduction of organ size/mass was also observed in co-suppressed R₀ tobacco plants.

Multiple co-suppressed lines also exhibited early flowering. Plants of these lines displayed reduced numbers of rosette leaves and fewer days before bolting. Because early-flowering phenotype was not observed in loss-of-function *ant* mutants, co-suppression by the *ANT* transgene could also influence other unknown *ANT* related genes that regulate flowering time by itself or together with *ANT*. Similar results were also observed in co-suppressed transgenic tobacco plants.

Example 4

This example shows that loss of *ANT* function reduces mature organ size by decreasing cell numbers.

Because *ANT* mRNA accumulated in leaf (Elliott, R. C., *et al.* (1996) *Plant Cell* 8:155-168), we examined the effect of a loss-of-function *ant* mutation on vegetative shoot development. While there was no difference in the timing of leaf primordia initiation or the number of leaf primordia between *ant-1* and control wild-type plants (not shown), the width and length of mature *ant-1* leaves were both reduced in comparison with those of corresponding wild-type leaves. Because *ant* mutant floral organs were found to be reduced in size (Klucher, K. M., *et al.* (1996) *Plant Cell* 8:137-153; Elliott, R. C., *et al.* (1996) *Plant Cell* 8:155-168), these observations demonstrate that loss of *ANT* function reduces organ size throughout shoot development.

A change in organ size can reflect an alteration in the size or number of cells, or both. To understand why *ant-1* organs are smaller, we examined the size and number of cells in mature *ant-1* organs and compared them with those in wild-type controls. The distal portion of the petal epidermis was observed initially because it has cells that are diploid and uniform in size and shape. We found that *ant-1* organs had fewer cells per unit area and per organ than wild type, however *ant-1* cells were much larger than normal. Essentially the same phenotype was observed in the epidermis and subdermal cell-layers of all *ant-1* floral organs and leaves. Thus, systemic reduction in size of *ant-1* organs is associated with a decrease in cell number, but not a decrease in cell size.

Because *ant* mutants reduce the number of floral organs, it has been suggested that *ANT* might be involved in organ primordium patterning as well as organ growth. To evaluate this possibility, we observed the pattern of sepal primordia in developing wild type and *ant-1* floral buds under SEM. By the end of floral stage 4

(Smyth, D. R., *et al.* (1990) *Plant Cell* 2:755-767), all four sepal primordia were initiated at the periphery of developing wild-type floral buds. In *ant-1* floral buds at the comparable stage, the organ primordia initiated were arranged normally in *ant-1* floral buds, although the number of floral organ was reduced (not shown). Thus, *ANT* appears to have little role in controlling the position of floral organ primordium in developing floral buds.

Example 5

This example shows the isolation of an *ANT* ortholog from *Brassica napus* (Canola).

The nucleic acid sequence and the encoded protein of the *Brassica* cDNA are shown in SEQ ID NO: 4 and SEQ ID NO:5 respectively.

To prepare this nucleic acid, total RNA was isolated from young shoot apices of *Brassica napus* (Canola) seedlings using TRIZOL as described by Colasanti *et al.* (*Cell*. 93:593-603 (1998)). cDNA was made by reverse transcription, and amplified by PCR using the high fidelity thermo-tolerant DNA polymerase PFU and oligonucleotide primers. The primers had the initiation codon and the anti-parallel nucleotide sequence downstream of the stop codon of the *Arabidopsis ANT* nucleotide sequence, respectively. The PCR products were subcloned into an *E. coli* vector and screened by PCR using different sets of oligonucleotide primers having internal *ANT* nucleotide sequence. Nucleotide sequence of the inserted *Brassica* DNA of selected recombinant plasmid clones was determined and compared to the *Arabidopsis ANT* nucleotide sequence for confirmation. The *Brassica ANT* (*BANT*) gene shares 85.5 % identity to the *Arabidopsis ANT* gene in their coding region at the nucleotide level and the *BANT* polypeptide sequence is 83.7 % identical to the *ANT* polypeptide sequence, respectively.

Example 6

This example shows use of the *ANT* 5'-upstream nucleotide sequence (promoter) for expressing heterologous genes in meristematic cells

A *HindIII*-*BglIII* fragment which includes the correctly oriented *ANT* promoter was inserted into the pBI101 plasmid vector DNA (CLONTECH) at the *HindIII* and *BamHI* sites which are located right before the initiation codon of the *GUS* (beta-glucuronidase) gene. The same fragment was also inserted into the plasmid pBIN m-

gfp5-ER (Haseloff, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:2122-2127, (1997) at the *HindIII-BamHI* sites located immediately before the initiation codon of the *GFP* (green fluorescence protein) gene. *Arabidopsis* wild-type plants were transformed by these recombinant plasmids using the *Agrobacterium*-mediated vacuum infiltration method.

- 5 Multiple T₁ lines, and their following generations, exhibited GUS activity or GFP expression in meristematic cells throughout plant development as expected, proving that the *ANT* promoter is useful for expressing a heterologous gene in meristematic cells.

Example 7

- 10 This example shows activation of the cyclin D3 (*CYCD3*) gene expression by increasing *ANT* gene expression in *Arabidopsis* plants.

- Cell proliferation is directly controlled by the activity of cell cycle regulatory genes, such as cyclins and cdks (Nasmyth, *Trends Genet.*, 12:405-412, (1996); Morgan, *Nature*, 374:131-134, 1995; and Burssens, *et al.*, *Plant Physiol. Biochem.*, 36:9-19, (1998)). Because organs from T₁ transgenic lines in which *ANT* gene expression is controlled by the CaMV 35S promoter had increased number of cells, and thus increased cell proliferation activity, expression of cyclin genes in young and mature organs of T₁ plants was measured by quantitative RT-PCR analysis. In young developing organs, where cell proliferation was observed in both 35S::*ANT* and control plants, the difference of expression levels of cyclin genes between them were not significant. However, in mature organs, while mRNA accumulation of *CYCD3*, which encodes a key regulator for G1/S entry in the *Arabidopsis* cell cycle (Soni, *et al.*, *Plant Cell.* 7:85-103 (1995); Fuerst, *et al.*, *Plant Physiol.* 112:1023-1033 (1996), is no longer detected in control, it was detected in 35S::*ANT* lines. These results agree with observations that no growth differences were detected at early stages of organ development between 35S::*ANT* lines and control lines; however, when organs of control plants were mature and ceased cell proliferation, cells in the same aged organs of 35S::*ANT* plants continued to proliferate and give rise to enlarged organs as the result.

- 30 This result demonstrates that the increased constitutive *ANT* activity directly and/or indirectly controls the cell cycle machinery via regulating expression of a cell cycle regulator gene(s) and continuously activating cell proliferation in developing organs. This also indicates that certain genes involved in cell cycle machinery are targets of the *ANT* transcription factor gene (Klucher *et al.* and Elliot *et al.*). Taken together,

these results suggest that modulation of expression of these ANT-target genes could regulate organ size/mass and fertility in plants.

Example 8

5 This example shows that ectopic expression of *BANT*, an *ANT* ortholog from *Brassica napus* (Canola), increases organ mass/size in *Arabidopsis*.

The Brassica ANT (BANT) cDNA, which has the nucleic acid sequence shown in SEQ ID NO:4, was inserted into the plasmid vector pMON530 (Rogers, et al., *Method. Ezymol.* 153:253, 1987) under the constitutive 35S promoter in the sense direction. The recombinant plasmid DNA was used for Agrobacterium transformation, and the Agrobacterium cells transformed with the 35S::*BANT* plasmid DNA was used for Agrobacterium mediated plant transformation by vacuum infiltration with *Arabidopsis* plants (Col-0 ecotype). T₁ seeds were collected about three weeks after vacuum infiltration, and planted on MS agar plates with kanamycin for screening T₁ transgenic seedlings.

T₁ plants ectopically expressing the 35S::*BANT* transgene exhibited multiple organ hyperplasia, as seen in 35S::*ANT* transgenic plants described above (Example 1). That is, leaves and floral organs were, at most, three times larger than control organs. These transgenic plants were essentially male sterile, and are often female sterile as well. Some plants, however, produced seeds upon fertilization with wild-type pollen grains by hand-pollination, and the T₂ seeds exhibited increased mass/size. The kanamycin-resistant T₂ seedlings developed into plants displaying the same phenotype as the T₁ plants, suggesting that the effect of *ANT* ectopic expression is heritable.

Example 9

This example shows increased *ANT* expression induces asexual reproduction and formation of adventitious shoots, organs, and embryos in *Arabidopsis* plants.

30 Fully matured stems or organs, such as leaves, were dissected from T₁ plants ectopically expressing *ANT* and placed in water or on MS agar plates without any phytohormones. After about two-week incubation, adventitious root formation was

observed at the cut surface of stems or leaves. Occasionally, adventitious roots were also produced from the leaf surface. This adventitious root formation was never observed control stems or leaves treated in the same way.

Excised inflorescence (flowering) stems from fully matured T₁ plants
5 ectopically expressing *ANT* were placed on MS agar plates without phytohormones for 10 days. Adventitious root formation was observed in the cut surface of stems, while adventitious shoot formation was observed in the senesced floral buds. These shoots eventually produced roots as well, developing into complete plants that exhibited the same transgenic trait (enlarged organ size/mass) as the original plants. The control
10 inflorescence stems did not show any activity of asexual reproduction under the same conditions.

Similar asexual reproduction was observed in embryos excised from developing *35S::ANT* transgenic seeds. The late torpedo-stage to nearly mature embryos were excised from developing green seeds, and grew on phytohormone-free MS agar
15 plates containing 50 µg/ml kanamycin. Although these embryos developed into seedlings, some cells reproduced secondary embryos or adventitious shoots, which also developed into complete plants. The control embryos did not propagate asexually under the same conditions.

CONCLUSION

20 In higher plants intrinsic organ size is determined genetically, although it can be influenced greatly by environmental factors. The size of organs reflects the number and size of cells. The total cell number of an organ is determined by the proliferation of undifferentiated meristematic cells that are competent to divide. During shoot development, lateral organs are initiated as primordia from apical and lateral
25 meristems. While most cells in organ primordia are meristematic and proliferate, cells lose meristematic competence and withdraw from the cell cycle as organs develop. Thus, the maintenance of meristematic competence of cells is a key mechanism that mediates organ growth and cell proliferation by defining total cell numbers, and thereby the size of plant organs. However, the molecular nature of meristematic competence and the
30 developmental regulators that control meristematic competence are not well understood.

The *Arabidopsis* *ANT* gene encodes a transcription factor of the AP2-domain family that has been found only in plant systems. *ANT* mRNA accumulates in primordia of all lateral shoot organs and diminishes as organs develop. This suggests that *ANT* may have a general function in organ growth. Consistent with *ANT* expression in leaf primordia and undifferentiated growing leaves, it was found that all mature leaves of the loss-of-function *ant-1* mutant were reduced in size in comparison with corresponding wild-type leaves. Because *ant-1* floral organs were also smaller than normal, *ANT* is most likely required for organ growth throughout post-embryonic shoot development. Organ size can be influenced by cell size, cell number, or both. It was found that *ant-1* organs had fewer cells per unit area and per organ than wild type, however *ant-1* cells were much larger than normal. This demonstrates that the systemic reduction in size of *ant-1* organs is the result of a decrease in cell number, but not a decrease in cell size. Therefore, *ANT* function is necessary to attain the intrinsic cell number of plant organs.

The experiments described here demonstrate that ectopic *ANT* expression is sufficient to increase organ size and mass by enhancing organ growth that is coordinated with organ morphogenesis in *Arabidopsis* plants. Differentiated cells in fully mature *35S::ANT* petals were the same size as those in wild-type petals. Similarly, no obvious difference in cell size was detected in the epidermis between control and *35S::ANT* organs other than petals. Thus, an increase of cell numbers, and not cell size, is primarily responsible for the enlarged *35S::ANT* organs. Similar loss- and gain-of-function effects on organ size was observed when plants were grown under short-day, continuous-light conditions, and in poor or rich media. Thus, *ANT* function seems to be independent of the perception of external growth signals. In contrast to the striking effects on final organ size, ectopic *ANT* expression did not perceptibly alter the size or structure of apical and lateral meristems, nor did it change the size or number of organ primordia. Although loss of *ANT* function reduced the number of floral organs, the organ primordia initiated were arranged and sized normally in *ant-1* floral buds. Therefore, *ANT* does not determine organ primordium size, and most likely does not influence organ primordium number by controlling the organization of the apical and lateral meristems.

How does *ANT* control cell numbers during organogenesis? In general, plant organ growth involves neither cell migration nor cell death; thus, organ cell

number essentially depends on proliferation of the meristematic cells in developing organ. Because *ANT* is expressed in meristematic cells of the developing organs, it might modulate cell proliferation during organogenesis and thereby determine the total cell number in mature organs. To test this idea, the extent of cell proliferation in control and
5 *ant-1* organs was tested by measuring cell numbers and cell size of both developing and fully mature petals. During mid-floral stage 9, the adaxial epidermal cells of wild-type petals were not differentiated and divided frequently, whereas *ant-1* petals had fewer undifferentiated cells than normal per unit area and per organ. This reduction in cell numbers became more pronounced in fully differentiated *ant-1* petals at stage 15. Thus,
10 there are fewer cell divisions than normal in *ant-1* petals throughout organogenesis, particularly during later developmental stages prior to maturation. Cell growth occurred without cell division in *ant-1* petals, resulting in extremely large cells.

These results suggest that *ANT* is required for the normal extent of cell proliferation, but not primarily for cell growth. To understand how *ANT* regulates the
15 extent of cell proliferation, we studied how ectopic *ANT* expression affects organ size, cell size, and cell numbers during petal development. In contrast to the early effect on cell numbers in *ant-1* petals, cell numbers and cell size in *35S::ANT* petals at stage 9 were normal. This demonstrates that ectopic *ANT* expression does not increase cell growth or the frequency of cell proliferation in developing petals during early stages, and suggests
20 that increased *ANT* activity does not alter the intrinsic cell cycle time. By stage 15, however, the total cell number of fully mature *35S::ANT* petals reached approximately 2.5 times that of controls, indicating that additional cell divisions occurred in *35S::ANT* petals prior to organ maturation, yet only after stage 9. Extra cell divisions must be coordinated with cell growth, since cell size in mature *35S::ANT* petals is normal.
25 Therefore, it is likely that ectopic *ANT* expression allows petal cells to proliferate for a longer period than normal without altering the intrinsic cell cycle time. Similar results were obtained when comparing growth of rosette leaves of *35S::ANT* and control seedlings. At 16 days after germination (16 DAG), both *35S::ANT* and control seedlings had the same number of rosette leaves, and all leaves of *35S::ANT* seedlings were the
30 same size as corresponding control leaves. However, *35S::ANT* leaves continued to grow beyond the period in which corresponding control leaves ceased to grow, eventually giving rise to larger leaves than normal. This observation supports the hypothesis that

prolonged cell proliferation coordinated with cell growth causes hyperplasia in *35S::ANT* plants. Taken together, these observations suggests that ANT regulates the period of cell proliferation by maintaining meristematic competence of cells during organogenesis. The results presented here also suggest that *ANT* does not influence *CycD3* expression in tissue where most cells are meristematic. Similar results were obtained in comparing mRNA levels of *CycB1b* (*Cyc1bAt*), a mitotic cyclin gene. Hence, ANT maintains the meristematic competence of cells, and consequently sustains expression of cell cycle regulators.

Another striking finding that connects *ANT* function with the maintenance of meristematic competence is neoplasia found in the *Arabidopsis 35S::ANT* organs. That is, clusters of undifferentiated cells (*i.e.*, calli) were generated from wounds or senesced-surfaces of *35S::ANT* plants, or detached-ends of fully differentiated *35S::ANT* organs without external phytohormone treatment. These calli often differentiated into organs, such as roots, leaves, or shoots. This neoplasia was observed consistently in *35S::ANT*organs, but never was seen in control organs treated in the same way. It is well established that differentiated plant tissue can induce calli after phytohormone treatment. Ectopic *ANT* expression in differentiated cells that are normally quiescent preserves meristematic competence and decreases their dependence on phytohormones for reentry into the cell cycle.

The findings presented here demonstrate ANT is an intrinsic organ size regulator that influences organ growth and the period of cell proliferation during organogenesis. In a proposed model of ANT action in plant organ size regulation, developmental growth signals activate growth regulators, which positively regulate ANT during organogenesis. ANT functions to maintain meristematic competence of cells, thereby modulating the expression of cell cycle and cell growth regulators. As a result, ANT sustains cell proliferation that is coupled to cell growth in developing organs. Ectopically expressed *ANT*, therefore, results in the abnormal retention of meristematic competence of cells and causes hyperplasia and neoplasia, while the absence of ANT causes precocious termination of cell proliferation and organ growth. In plant and animal systems, growth signaling pathways and the cell cycle machinery appear to share many common factors. Nevertheless, given the immobile attributes of plant life and plant cells, which are surrounded by rigid cell walls, some aspects of plant growth and cell

proliferation are likely to be regulated and coordinated in a different way from those of animals. Thus, it may not be surprising that *ANT* is a plant specific regulator, and identification of upstream regulators and downstream targets of *ANT* may reveal how plants uniquely coordinate cell proliferation with pattern formation to control organ size.

- 5 It has been suggested that the genetic basis for plant interspecies diversity of phenotype might be minor changes in the structure or expression of orthologous regulatory genes. Hence, differences in structure and expression pattern of *ANT* and its orthologs, at least in part, may be a mechanism that is responsible for the interspecies diversity of organ size in higher plants. Finally, increasing organ mass by ectopic *ANT* expression might be a new
10 method for improving the yield of agriculturally important plants.

- The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents,
15 and patent applications cited herein are hereby incorporated by reference for all purposes.

SEQUENCE LISTING

SEQ ID NO:1 [GenBank No. U40256]

Arabidopsis ANT nucleotide sequence (cDNA:2148 nucleotides)

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1 agatccaac ggattcaaac agcaaattg tgcttgctc ttctctctta ttataatc
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SEQ ID NO:2

10 [GenBank No. U40256] *Arabidopsis* ANT peptide sequence (555 amino acids)
 MKSFCNDNDNNHSNTTNLLGFSLSNMMKMGGRGGREAIYSSST
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 15 NQNQNHQSQNHQQISEALVETSVGFETTTMAAAKKKRGQEDVVVVGQKQIVHRKSIDT
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 AALKYWGPSTHTNFAENYQKEIEDMKNMTRQEYVAHLRRKSSGFSRGASIYRGVTRH
 HQHGRWQARIGRVAGNKDLYLGTFTGTQEEAAEAYDVAAIKFRGTNAVTFNFDITRYDVD
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 20 LPQVNQKMFSGSNMGGNMSPWTSNPNAELKTVALTLPQMPVFAAWADS

SEQ ID NO:3

ANT promoter sequence (5'-upstream sequence of the *ANT* gene:4228 nucleotides)

Underlined nucleotide sequence was published (Klucher *et al.*)

25 5'-
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 A
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 GTAATTTATCTTAAACCAAAGTTTGAATTGTGCATTGGGAGCTACACTCTAGTCCCTTTTTTCCCCAAAAATAATCTCC
 35 TTACATCGACCGGTTAAAGTATTTAAACCAACAAATTTTAATTGTTGCTGAAGGTACAAACATGTCACATATATAGAG
 A
 CAGCATCGTTTATACAAATAATGTTTCGATGTTATTGGAAATCAAAATATAAATACGAATTAGCGACTCACTTGGTTAATA
 GTTTGGAAAGATAATGAAATAAAAAATGAATTCAAAGGATACAGAGCTATATATGTCGGGTCAATTAGAGCCGTGACCA
 AA
 AGTTTCGTGTAATTTCTACGGTCGGTCATAAGAAAATTTGGACTTTTCTTACCCTTTTATGAAC TCTGTATAGTTT
 40 TGTCGGATTATATATTGTATTCGTATATTTTTGTTTCTAATAATGATACGTAAATTCACGATAAGAAAGACTTCTTTT
 TATTTAATTTGATTTAAAC TTTTGT TTTTGGAAATGACTCATACACAAGGTTAAAGTTTGATGGTATCCAATTTACAA
 AATGTTTCGAGAGTGCGTTTCGAGTGTCTACCACCATCGTACCAACTCGTATGGGTTTATTATTAAGT TTTTCTCTT
 TTTCCAATGTCTTTATAATTGAACCACTCTAAATTTCTTTTTTAAATTAGGTTAAGAATCTTGAATTTTCTGTGATT
 45 TAAACCAAGGTTTCAATTTCTTCTAGCACAAAAAAGGTTTCAATTATTAAGAATCTAAATTTTTTGAGT
 TCAAGAGTTAATGATAGCTGAAAAGTTATGAATGATTGCAAGTTTGCAACAGAATGGTCGATGTAGTACATATCAAAA
 A

CATGCATCAAAATAAATATTCGTGCTTAGCAAGAGAAACGATTGAAATAAACAGAACAAATCGTTAACCACTTAAAAAT
CT
TAGAATAATTTTGTAGTGATAATTTTCTGTAAGAGAGAGGTATCATATCTTACAAAAAAAACCTATTTTCAGATAAAAT
A
5 ATGTTGTCCAATCGTTACCAAGTATGTTTTGCTGTCATCAGTTGTATTGTAACCTCGTCTCTTAGCCATATAGTTCTAAG
TTTTAAATGTTTTCAAAGACTTTACAAAAATAAAATAATAAAGGTGGAATTTGTAGGGCTAAAAGCGAAAAATAAA
AA
TAAATAAAAGTAAAGAAACGTCTTTCTCAATAAGAACACAGATCCCAACGGATTCAAACAGCAAATTTGTGCTTTGCT
C
10 TTCTCTCTTATTATAATATCCTCTCAAAAACCTCTCCTATATCCTCCTAAAGCCCCCTTCCTTGTTTCTCTACCGCAA
CAAAGAAAAAACAAAAGTTTGAGAAAAATGGTGTGTTCTGTTGTGAACCAATGATTGGGTTTTAGCTTACTACTTCGAG
A
GATTATAAGAAAGAAAGAGTGAAGATACATTATAGAAAGAAGAGAAGCAGAAACCAAAAAAGAAACC -3'

15 SEQ ID NO:4

Brassica ANT nucleotide sequence (partial cDNA including coding region: 1738 nucleotides)

5'-

ATGAAGTCTTTTTGTGATAATGATGATAGTAATACGACTAATTTGCTAGGGTCTCGTTGTCTTCAAATATGTTGAAAAAT
20 GGGTGGTGGAGAAGCTCTTTACTCATCTTCGTCGCTTCAGTTGCAACTTCTTCTGTCCACCACAGCTTGTTGTGGCG
ACAACAGTAGCAACTATGGAGTTTGCTACGGTTCTAACTTAGCAGCTAGGGAAATGTATTCTCAAATGTCTGTGATGCC
C
CTCAGATCTGACGGTTCTCTTTGCTTAATGGAAGCTCTCAACAGATCTTCTCACTCGAATAATCATCACCATAGTCAAGT
TTCATCTCCAAAGATGGAAGATTTCTTTGGGACCATCATCACAACACAAGTCACAAAGAAGCCATGGATCTTAGCTTA
25 G
ATAGTTTATTCTACAATACCACTCATGCGCCAAACAACAACACCAACTTTCAAGAGTTCTTTAGCTTCCCTCAAACCTAGA
AACCACCATGAGGAAGAAACAAGAACTACGAGAATGACCCTGGTTTGACACATGGAGGAGGGTCTTTAATGTAGGG
GT
ATATGGGGAATTTCAACAGTCACTGAGCTTGTCATGAGCCCTGGGTCACAATCTAGCTGCATCACTGCCTCTCATCACC
30 ACCAAAACCAAACCTCAAACCAACAGCAGATCTCTGAAGCTTTGGTCGAGACAAGTGCTGGATTGAGACAACAACAA
TG
GCGGCTGCTGCTGCAAAGAAGAAGAGAGGACAAGAAGTTGTCGTTGGACAGAAACAGATTGTTTCATAGAAAACTATT
GA
TACTTTTGGACAACGAACTTCGCAATACCGAGGCGTTACAAGACATAGATGGACTGGTAGGTATGAAGCTCATCTATGG
35 G
ACAATAGTTTCAAGAAGGAAGGTCATAGCAGAAAAAGGAAGACAAGTTTATCTGGGGGGTTATGATATGGAGGAGAAA
GCT
GCTCGAGCATATGATCTTGCTGCACTCAAGTACTGGGGTCCCTCTACTCACACTAATTTCTCTGTGGAGAATTATCAGAA
AGAGATTGATGACATGAAGAACATGACTCGACAAGAATATGTTGCTCACTTGAGAAGAAAAACAGTGTTTCTCTAG
40 GG
GTGCTTCCATCTATAGAGGAGTCACCAGACATCACCAGCATGGAAGGTGGCAAGCTCGGATCGGTAGAGTCGCTGGAA
AC
AAAGATCTCTACCTTGGAACCTTCGGAACCTCAAGAAGAAGCGGCGGAAGCCTATGATGTAGCAGCTATCAAGTTCCGTG
G
45 CACAAACGCGGTGACTAACTTTGACATAACAAGGTACGATGTTGATCGCATAATGGCTAGTAACACTCTTGTCTGGA
G

AGATGGCTCGAAGGAACAGCAACAGCATCGTGGTCCGCAACATTAGCGACGAGGAAGCCGCTTTAACCGCTGTCGTGA
AC
GGTGGTTCCAATAAGGAAGTGGGTAGCCCGGAGAGGGTTTTGAGTTTTCCGACGATATTTGCGTTGCCTCAAGTTGGTC
C
5 GAAGATGTTCCGAGCAAATGTGGTCGGAAATATGAGTCTTGGACTACGAACCCTAATGCTGATCTCAAGACCGTTTCT
C
TTACTCTGCCGAGATGCCGGTTTTCGCTGCGTGGGCTGATTCTTAATTCAATCTAATGGCTAACTCTGGTTTTCTTGGTT
TAGGGTCCAAGTGTTAAGTTTATCTCCGGGTTTATCCGGTTTGAACAATTCGG-3'

10

SEQ ID NO:5

Brassica ANT peptide sequence (548 amino acids)

MKSFCDNDDSNTTLLGFSLSNMLKMGGGEALYSSSSSVATSSVPPQLVVGDNSSNYGV CYGSNLAAREMYSQMSVMP
LRSDGSLCLMEALNRSSHNNHHHSQVSSPKMEDFFGTHHHNTSHKEAMDLSLDSL FYNTTHAPNNNTNFQEFFSFPQTR
15 NHHEEETRNYENDPGLTHGGGSFNVGVYGEFQQSLSLSMSPGSQSSCITASHHHQNQTQNHQQISEALVETSAGFETTM
AAAAAKKKRGQEVVVGQKQIVHRKSIDTFGQRTSQYRGVTRHRWTGRYEAHLWDNSFKKEGHSRKGRQVYLGGYDMEEK
A
ARAYDLAALKYWGPSTHTNFSVENYQKEIDDMKNMTRQEYVAHLRRKTSGF SRGASIYRGVTRHHQHGRWQARIGRVAG
N
20 KDLYLGTFGTQEEAAEAYDVA AIKFRGTNAVTFNFDITRYDVDRIMASNTLLSGEMARRNSNSIVVRNISDEEAALTAVVN
GGSNKEVGSPERVLSPFTIFALPQVGPKMFGANVVGNMSSWTTNPNADLKT VSLTPQMPVFAAWADS

SEQ ID NO:6

25 Consensus *ANT* polynucleotide sequence-1 (33 nucleotides)

ATGAAGTCTTTTTGTGATAATGATGATAGTAAT

SEQ ID NO:7

Consensus *ANT* polynucleotide sequence-2 (39 nucleotides)

30 ACGACTAATTTGTTAGGGTTCTCATTGTCTTCAAATATG

SEQ ID NO:8

Consensus *ANT* polynucleotide sequence-3 (38 nucleotides)

AGAATCAGCCCAAGCAGCGAAAACCGGCATCTGCGGCA